Problems in the Laboratory Diagnosis of Rabies*

W. D. STOVALL, M.D., F.A.P.H.A., AND S. B. PESSIN, M.D.
State Laboratory of Hygiene, Madison, Wis.

THE diagnosis of rabies in the laboratory is based entirely upon the microscopic demonstration of Negri bodies and upon animal inoculation. The demonstration of Negri bodies is the method of choice since the diagnosis can be thus made in a few minutes or hours. When the technic employed demonstrates typical bodies the result is highly convincing and satisfying. However, negative and doubtful results leave much to be desired, and animal inoculation must be resorted to. The difficulties in demonstrating Negri bodies arise from two sources of error which can be enumerated as inability to differentiate them from other inclusion bodies and cell structures, and inherent deficiencies in the methods of examination.

NEGRI BODIES, LYSSA BODIES, AND OTHER BODIES

The large Negri body with the inner structure well developed and stained is easily recognized by any microscopist who has seen a few demonstrations. No long years of experience are required since the difficulty lies not in the recognition of the typical body but with its demonstration. Negri [1] described the body as occurring in two forms, one in which the inner bodies are small and highly refractile and another in which they are larger, less refracting, roundish, oval, or irregular. In one phase of their development the cytoplasm encloses a mass of minute inner bodies which when they are distributed through the central nervous system in single units cannot be differentiated from the granular structure of the cellular elements. Since the identity of the Negri body is not yet understood, its development is not known. However, every experienced microscopist has encountered the difficulty of deciding whether the bodies observed in some preparations are Negri bodies or cytoplasmic structures normal to the cell or if not normal at least only distorted cellular structures. Goodpasture [2] refers to the variation in size of Negri bodies and speaks of being able to demonstrate the smallest forms. When small bodies are associated with large ones, which show the typical inner structure, no confusion is encountered. When, however, only forms so small occur that the demonstration of the "Innenkörper" is doubtful, the diagnosis is doubtful. The brain of cats, particularly, offers difficulty because of the pink staining granular material in the cells and also because the Negri bodies in the pyramidal and Purkinji cells of this animal are often very small. The failure of the microscopic diagnosis of rabies as proved by mouse inoculation is shown in Table 1.

It is not always because of the size of Negri bodies that satisfactory demonstration fails. There do occur in

* Read before the Laboratory Section of the American Public Health Association at the Seventieth Annual Meeting in Atlantic City, N. J., October 16, 1941.

TABLE 1

Rabies—Results of Mouse Inoculations from Oct. 29, 1937, to Sept. 17, 1941

Type of Animal	*Dog*	*Cow*	*Skunk*	*Horse*	*Fox*	*Hog*	*Squirrel*	*Groundhog*	*Rabbit*	*Monkey*	*Rat*	*Cat*	*Sheep*	*Muskrat*	*Chicken*	*Total*
Number examined	300	41	13	14	11	7	10	4	2	2	5	29	2	1	1	442
— By smear and sections	250	31	7	9	3	6	10	3	2	2	5	22	2	1	1	354
— By smear and sections — By mouse inoculation	226	20	6	5	3	3	10	3	2	2	5	21	2	1	1	310
— By smear and sections + By mouse inoculation	24	11	1	4	0	3	0	0	0	0	0	1	0	0	0	44
% + Discovered by mouse inoculation	9.6%	35.5%	14.3%	44.4%	0	50.0%	0	0	0	0	0	4.5%	0	0	0	12.43%
+ By smear and sections	43	10	3	5	7	1	0	0	0	0	0	7	0	0	0	76
+ By smear and sections + By mouse inoculation	39	9	3	4	7	1	0	0	0	0	0	3	0	0	0	66
+ By smear and sections — By mouse inoculation	4	1	0	1	0	0	0	0	0	0	0	4	0	0	0	10
% Error	9.3%	10.0%	0	20.0%	0	0	0	0	0	0	0	57.1%	0	0	0	13.16%
Unsatisfactory for smear and sections	7	0	3	0	1	0	0	1	0	0	0	0	0	0	0	12
Unsatisfactory as above — By mouse inoculation	6	0	2	0	0	0	0	0	0	0	0	0	0	0	0	8
Unsatisfactory as above + By mouse inoculation	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	4

— Negative + Positive

rabid animals bodies described by Goodpasture [2] as "lyssa bodies." These bodies differ from Negri bodies in size and the absence of "Innenkörper." They are described as very small, pink staining, homogeneous spheres. They are found with and without recognizable Negri bodies. In those brains containing no unmistakable Negri bodies these bodies are the cause of much confusion in the diagnosis. Animal inoculation is the only method by which the diagnosis in such cases can be made. Because the microscopic diagnosis was indefinite in some cases we injected mice with material from 76 animals which had been reported as Negri-positive or doubtful. As shown in Table 1, only 66 of these animals proved to have rabies by mouse injection. Additional experience with this discrepancy does not assist in its prevention since we find instances where rabies virus is associated in the brain with eosinophilic bodies that are not Negri bodies.

As a result of this experiment we were prompted to examine for eosinophilic bodies the brain from apparently well cows and sheep that were killed at a local packing plant. Sections were prepared from portions of the hippocampus, cerebral cortex, cerebellum and medulla. In the medulla and cerebral cortex we were able to demonstrate large and small bodies which took the eosin stain. These bodies were both intracellular and extracellular. They were for the most part homogeneous but sometimes there was a small dark staining granule in the center. These bodies resemble "lyssa bodies" more than Negri bodies. Since Goodpasture demonstrated that "lyssa bodies" are homogeneous, and since bodies demonstrated in the brain tissue of normal animals and animals suspected of having had rabies have the same homogeneity and staining reaction, even the most experienced observers cannot make a decision without mouse inoculation.

SMEARS—IMPRESSIONS AND SECTIONS

Sellers [3] reported that 40 of 46 laboratories questioned about the methods employed to demonstrate Negri bodies used smear or impression. Only 2 laboratories used paraffin sections. When typical Negri bodies are present these preparations are satisfactory. In our experience typical Negri bodies are often scarce and difficult to demonstrate in the brain tissue of hogs, cows, horses, and cats. In paraffin sections hundreds of cells can be examined quickly, and in properly fixed and stained preparations the sections are clear and there is good visualization of the structures of the cells as well as small Negri bodies. The importance of the examination of large numbers of cells from different parts of the brain and cord is emphasized by the wide distribution of the virus in the brain. In a study of 84 Negri-positive brains we found Negri bodies in the cerebral cortex alone in four instances (Table 2). In a comparison of the direct smear method with paraffin sections we found that in 10 per cent of the cases the sections were Negri-positive when the smears were negative. In no case were the sections negative when the smears were positive.

TABLE 2

Distribution of Negri Bodies in Brain

	Brains
Hippocampus only	8
Hippocampus and cerebral cortex	13
Hippocampus, cerebrum and cerebellum	24
Hippocampus, cerebrum cerebellum and medulla	31
Hippocampus, cerebrum and medulla	1
Hippocampus and cerebellum	3
Cerebral cortex only	4
Total	84

The chief objection to sections is that they are time consuming and offer more technical difficulties than smears do. However, the delay in the diagnosis occasioned by sections is not significant. At the longest it is never more than a few hours. When the incubation period of the disease is considered, even the

matter of a day is not important. If the acetone fixation described by one of us (Stovall [4]) is used, the sections are ready for examination in 3 to 5 hours. The small pieces of tissue can be left overnight in the acetone but they are ready for embedding in paraffin within 2 to 3 hours, depending upon the size of the pieces of tissue. It is not unusual for us to have the sections ready for examination in the late afternoon when specimens are received in the forenoon. It is our practice to prepare smears as soon as the specimen is received, and if they are Negri-negative, sections are examined before animals are inoculated. The technic is not complicated or laborious, and the equipment is nothing more than is found in most laboratories.

STAINS

In our hands eosin-methylene blue is the most satisfactory stain for the demonstration of Negri bodies in routine diagnostic work. One of us has shown the importance of dissolving the dyes in a solution in which a low pH is maintained. The study showed that 1 per cent eosin (yellowish) in 95 per cent alcohol maintained at pH 6 stains Negri bodies a pale pink; at pH 3.0 the large and small Negri bodies stain a uniform deep red. The counter stain was methylene blue adjusted between pH 5.0 and pH 6.0. As the pH of the methylene blue rises above pH 6.0, more and more of the eosin is removed from Negri bodies until at pH 8 the small bodies cannot be seen and the large and more typical ones are pale and atypical. This destaining action of the methylene blue solution as it approaches neutrality seriously interferes with the visualization of Negri bodies. When eosin and methylene blue are maintained at the optimum pH the cytoplasm of the nerve cells takes a diffuse pale blue color and the body of both the small and large Negri bodies a deep red. The inner structure of the Negri bodies appears as dark blue granules. In the small ones there is often only one small granule. The " lyssa bodies " are a homogeneous red color.

Sellers [5] has recommended basic fuchsin and methylene blue in an alcoholic solution for smears and impressions. Because we have found eosin-methylene blue used as described above so satisfactory for paraffin sections, we have also used it for smear preparations.

ANIMAL INOCULATION

Above we have mentioned the occasional occurrence of what appear to be " lyssa bodies " or small Negri bodies in the brain of some animals which did not produce rabies when injected into mice. These bodies are found most frequently in the cerebrum and medulla. Since in the study of 84 cases of rabies proved by mouse inoculation we found Negri bodies only in the hippocampus 8 times and only in the cerebral cortex 4 times (Table 2), the finding of *eosinophilic bodies* in any portion of a brain from an animal suspected of having had rabies creates a doubt as to the diagnosis. In all such cases we have injected mice, as well as in all cases that were clearly Negri-negative. Thus in Table 1 it will be seen that of 354 animals found to be Negri-negative or doubtful only 310 were rabies-negative by mouse inoculation, giving a failure in the microscopic diagnosis of 12.43 per cent. While this is in apparent close agreement with the report of Leach,[6] it may not be comparable. There are several reasons for this. In the first place the total number of examinations made by us is less than the number made by Leach. Probably more important than this is the difference in the variety of animals represented and the number of each variety.

Sometimes specimens are received that are badly decomposed and for that reason they have been unsatisfactory for microscopic examination.

The intracerebral injection of mice with contaminated brain tissue invariably causes death of the animals from bacterial infection. To avoid this Sulkin and Nagle [7] investigated a number of bactericidal agents in which they immersed the contaminated tissue. They did not try filtration because reports indicated that this method was unsatisfactory for a health department laboratory. However, we have used filtration through Seitz filters, size No. 3, with all specimens showing contamination or which were decomposed. Mice inoculated with filtrates from typical Negri-positive brains have not failed to develop rabies. The organism which invariably causes purulent meningitis in mice is a small Gram-positive bacillus. We have not had time to work with this organism, and so far we have made no attempt to identify it. In our hands the Seitz filter removes these bacteria and the filtrates are highly satisfactory for intracerebral inoculation of mice.

Small portions of hippocampus, cerebellum, cerebrum, and medulla are emulsified in 10 ml. of sterile saline. If the smears or sections reveal bacteria, the emulsion is passed through the filter and mice injected with 1/100 to 3/100 of a ml.

Of particular interest is the result of mouse inoculations with emulsions from 24 specimens in which small atypical inclusion bodies were found in the cerebral cortex. In 5 instances the bodies were considered characteristic enough to consider the microscopic examination to be Negri-positive. In only 1 of the 5 was rabies virus demonstrated. The remaining 19 specimens were considered doubtful by microscopic examination. Of these, 9 were rabies-positive and 10 were rabies-negative. From these results it appears that by microscopic examination of sections and in some smears we are able to demonstrate eosinophilic bodies resembling "lyssa bodies" and atypical Negri bodies which are not associated in the brain with rabies virus. Also the results show that brain specimens in which the microscopic examination leaves the diagnosis in doubt contain rabies. The bodies that cause this confusion in the microscopic diagnosis of rabies are similar to ones found in certain parts of the brain of normal cattle and other animals and to atypical or small Negri bodies.

REFERENCES

1. Negri, A. *Ztschr. f. Hyg. u. Infectionskr.*, 43:507, 1903.
2. Goodpasture, E. W. *Am. J. Path.*, 1:547, 1925.
3. Sellers, T. F. *Report to Conference of State and Provincial Laboratory Directors*, Oct., 1938.
4. Stovall, W. D., and Black, C. E. *Am. J. Clin. Path.*, 10:3, 1940.
5. Sellers, T. F. *Diagnostic Procedures and Reagents*, A.P.H.A. 1st Ed., 1941.
6. Leach, Charles N. *A.J.P.H.*, 28:162, 1938.
7. Sulkin, S. E., and Nagle, N. *J. Lab. & Clin. Med.*, 25:94, 1939.